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EFFECTIVITY OF FERMENTED GOAT MILK ADDED LACTOBACILLUS PLANTARUM AS MELANIN INHIBITOR

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ABSTRACT

This study aimed to investigate the affectivity of goat milk fermented with Lactobacillus plantarum (TW 14) as melanin inhibitor. Prior to the treatment of melanin inhibitor, samples were pasteurized. After that, the samples were incubated at 37 ^{o}C for 24 hour and analyzed for lactic acid bacteria population, total titratable acid, protein and fat contents. Fermented milk were extracted by centrifugation, the supernatant was collected and used for inhibition of tyrosinase enzymes activity and inhibitory melanin on B16F0 cell analysis. The results showed that the milk used complied with standard fermented milk which able to inhibit tyrosinase activity (L-DOPA Substrat). Goat milk was unable to inhibit melanin synthesis on B16F0 cell but fermented milk showed the ability, inhibit tyrosinase and melanin synthesis on B16F0 cell. A concentration fermented sample of 66.7 mg/ml resulted in a decrease in melanin concentration of extracellular in TW 14 treatment groups for 27%. In general goat milk fermented with lactic acid bacteria is an effective as melanin inhibitor.

1. INTRODUCTION

Dairy goat has been a prospective commodity to develop for several reasons. Relatively small investment is reqquired to build farms of this multipurpose commodity. Milk goat is also known as unperishable animal product fr its smaller globules which takes longer time to release free radical [1]. Consumers's preferences on milk goat is mainly because it is beneficial for health and beauty.

The application of fermented milk on skin is associated with the therapy process of wound and burns and recently becomes popular as cosmetic material sources [2]. Milk fermentation using *Lactobacillus helveticus* produces milk that can give good effect on skin moisturizing. In addition, lactic acid bacteria-fermented soy milk gives effect on suppressing melanogenesis in B16F0 melanocytes cell culture [3].

Melanin is essential for protecting human skin against radiation, but many reported about accumulation of abnormal melanin induces pigmentation disorders, such as melisma, hyperpigmentation, freckles and

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ephelides [4]. In mammals including in humans, tyrosinase is responsible for melanogenesis or hyperpigmentation [5]. Controlled of melanosit by tyrosinase enzyme and antioxidant. The formation of melanin in the human body is reduced by several mechanisms, including antiokxidation, direct tryrosinase enzyme, melanin inhibition of migration from cell to cell and hormonal activities, etc [6]. The enzyme is widely distributed in fungi, higher plants and animals , and is involved in the first two steps of the melanin biosynthesis, in which L-Tyrosine is hydroxylated to 3,4- dihydroxyphenylalanine (monophenolase activity) and the latter is subsequently oxidated to DOPA quinone (diphenolase activity). A large number of moderate to potential tyrosinase inhibitors from natural and synthetic resources have been reported during the last decade. Tyrosinase inhibitors such as arbutin, kojic acid and hydroquinone have been used as whitening or antihyperpigment agents because of their ability to suppress dermal-melanin production [7].

The application of lactic acid bacteria-fermented goat milk as cosmetic material source is still very limited, thus this research was designed. Furthermore, the objective of this study was to study the effectiveness of fermented goat milk as melanin inhibitor under enzymatic process and in B16F0 cell culture using *Lactobacillus plantarum* (TW 14) starter.

2. MATERIAL AND METHODS

2.1. Goat Milk Preparation

Goat milk used in this research was produced from Peranakan Etawa (PE) goat obtained from Daya Mitra Primata Cooperative, Cikarawang Village, Dramaga District, Bogor. The goat milk used in this research was freshly produced from morning milking time and then packaged in HDPE plastic. Pasteurization is a heating processing method below the boiling point for extending the shelf life of freshmilk. Low Temperature Long Time (LTLT) method used temperature of 62.8 ºC for 30 minutes [8].

2.2. Goat Milk Fermentation

Milk fermentation was carried out using lactic acid bacteria isolate i.e. *Lactobacillus plantarum* (TW 14). This bacterium was isolated from Peranakan Etawa goat milk collected from Daya Mitra Primata Cooperative, Cikarawang Village, Dramaga District, Bogor [9]. Lactic acid bacteria culture which added during fermentation process was 5.0% with incubation time of 24 hours. The observed parameters were total population of lactic acid bacteria, total lactic acid bacteria, protein level and fat level [10].

2.3. Milk Extraction

Sample of milk (10 g) were homogenized with 2.5 mL of sterile distilled water. The pH of the yogurts was determined and the sample subsequently acidified to pH 4.0 with HCl (0.1 M). The acidified sample were then heated in water bath (45 ^{o}C) for 10 min followed by centrifugation (5000g, 10 min 4 ^{o}C). NaOH (0.1 M) was added to adjust the pH of supernatant to 7.0. The neutralized supernatants were re-centrifuged (5000 g, 10 min ^{o}C) and the supernatant was harvested and stored in a min 20 ^{o}C freezer [11]. After that, the samples to freeze drying processed until required for analysis.

2.4. Measurement of Tyrosinase Inhibitor

Extracted milk sample was directly tested on drop plates. Kojic acid was used as positive control. A total of 70 µl of each extract was added with 30 µl of tyrosinase enzyme (Sigma 333 unit/ml in phosphate buffer) and then followed by incubation process at room temperature for five minutes. At each hole of multi plates, 110 µl substrate (2 mM L-Tirosine or 12 mM L-DOPA) was added and then incubated for 30 minutes at room temperature. The mixture was measured using multiplates reader at 492 nm of wavelength [12].

2.5. Cell Culture

Murine melanoma B16-F0 cells (DS Pharma Biomedical, Osaka, Japan) were cultured in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100,000 unit/L penicillin and 100 mg/L streptomycin. Cells were cultured at 37 ^{0}C in humidified atmosphere of 5% CO2 [13].

2.6. Measurement of cellular melanin content

Confluent cultures of B16 melanoma cells were rinsed in phosphate-buffered saline (PBS) and removed using 0.25% Trypsin/EDTA. The cells were loaded into a 24 well plate and allowed to adhere at 37 ^{0}C for 24 h. Sample prepared with concentration 66.7 mg/ml, 33.3 mg.ml and 16.7 mg/ml for 1-6 were added the cells incubated for 72 h. following incubation, cell medium was collected and 200 µl were loaded into a 96 well plate. The absorbance of the medium was measured at 510 nm using a microplate reader and used as measured at extracellular melanin content. The cells were washed with PBS following lysis in 600 µl of 1 M

NaOH by heating at 100^0C 30 min to solubilize the melanin. A portion of the resulting lysate (250 µl) was loaded into at 96 well micro plate, and the absorbance was measured at 405 nm using a micro plate reader. Measured absorbance as use an index of intracellular melann contents. Each experiment was repeated twice. The melanin producing activities were expressed as a percentage of the activity measured in the control cells treated DMSO without sample materials [14].

3. RESULT AND DISCUSSION

Milk without starter (STS) and milk with the addition of *Lactobacillus plantarum* (TW 14) lactic acid bacteria were incubated at 37 ^{0}C temperature for 24 hours after pasteurization processed. Observation was carried out on the population of lactic acid bacteria (BAL), total lactic acid (TAT), protein level (%) and fat level (%). Data is shown in Table 1.

Table 1 Goat milk quality for 24 hours incubation

Sample	BAL Population (cfu/ml)	TAT (%)	Protein (%)	Fat (%)
Milk (STS)	$8.3 \times 10^6 \pm 0.002$	$0.74^{b} \pm 0.201$	$7.02^{a} \pm 0.067$	$7.67^{a} \pm 0.022$
Milk + TW 14	$9.5 \times 10^7 \pm 0.012$	$1.44^{a} \pm 0.103$	$6.05^{b} \pm 0.013$	$6.50^{b} \pm 0.007$

Different superscript at same column shows significant different at $P < 0.05$ at $\alpha = 0.05$ level

Based on the observation result on goat milk and lactic acid bacteria-fermented goat milk, the population of lactic acid was 10^7 cfu/ml at TW 14. The population of lactic acid has significant effect on milk quality, the minimum number of lactic acid bacteria in fermented milk is 10^7 cfu/ml [15]. According to that number, this research had found that BAL population complied with the standard. Decreasing number of protein level in fermented milk was due to the activity of BAL catabolism protein into peptides.

As shown in Table 3, fat level also decreased at lactic acid bacteria-fermented goat milk. This could be due to the increasing of lactic acid during fermentation process which has lipolytic activity to reduce milk fat [1].

3.1. Tyrosinase Inhibitory Activity

Tyrosinase is an enzyme which has function on the formation of skin pigment. By supress the activity of tyrosinse, the formation of melanin could be inhibited. Tyrosinase inhibitory activity test was carried out using kojic acid as positive control which applied at two substrates i.e. L-Tyrosine and L-DOPA. The tyrosinase inhibitory activity of goat milk without starter (STS) and with fermentation by *Lactobacillus plantarum* (TW 14) are shown in Table 2.

Table 2 Tyrosinase inhibitory activity (%)

Sample	Substrate	
	L-Tyrosinase	L-DOPA
Milk (STS)	$33.196^{a} \pm 0.269$	$53.108^{a} \pm 0.026$
Milk + TW 14	$38.594^{a} \pm 0.086$	$45.892^{b} \pm 0.106$
Kojic Acid (62.5 ppm)	70.078	73.737

Different superscript at same column shows significant different at $P < 0.05$ at $\alpha = 0.05$ level

Both milks fermented, with and without starter could inhibit tyrosinase activity by the two substrates L-Tyrosine ((Monophenolase) and L-DOPA (Diphenolase). Milk quality added with TW 14 significantly in L-DOPA substrate. The Inhibition activity fermented of goat milk used starter and non starter are not as good as inhibitory activity of kojic acid at concenteration of 62.5 ppm as positive control.

Tyrosinase played important role in melanin formation during melanogenesis process due to the ability to hydroxylase L-Tyrosin (monophenol) to L-DOPA (phenol) and oxidize L-DOPA to DOPAquinon (quinone compounds). Formed DOPAquinone will spontaneously react to form DOPAkrom. Its role in melonogenesis process occurred as tyrosinase contains copper groups as an active site that can associate together with substrate in melanin formation process [16].

Research carried showed that fermented milk process for the melanin formation inhibition by using Lactobacillus plantarum M23 with tyrosinase inhibitory activity. This research used 4-factor-3-level central composite design combining with response surface methodology. Yeast extract concentration (%, X1), addition of grape (%, X2), incubation temperature (^{o}C, X3) and incubation time (h, X4) [17].

3.2. Cell Viability and Melanin Content

Measurement of cell viability and melanin content was performed in five different concentrations i.e. 16.7, 33.3 and 66.7 mg/ml. The decreasing number of melanin content in goat milk fermentation without starter is shown in Figure 1.Ddimethyl sulfoxide and arbutin as negative and positive control.

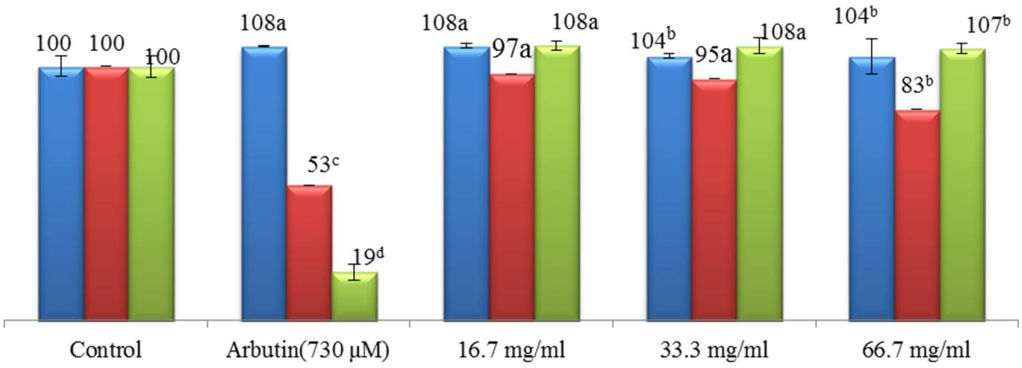

Figure 1 The decreasing number of melanin content in goat milk (STS)
: cell viability, : melanin intercellular content,
: melanin extra cellular content

The treatment with goat milk fermentation without lactic acid bacteria starter is not toxic to the cell because the cell viability higher than control (Fig. 1). Through the it could inhibit tyrosinase on diphenolase but not the are no inhibitory activity on formation of extracelluler melanin. Only small decreasing of sample of 66.7 mg/ml resulted melanin intracelluler (17%). The inhibitory resulted of TW 14 is shown in Figure. 2.

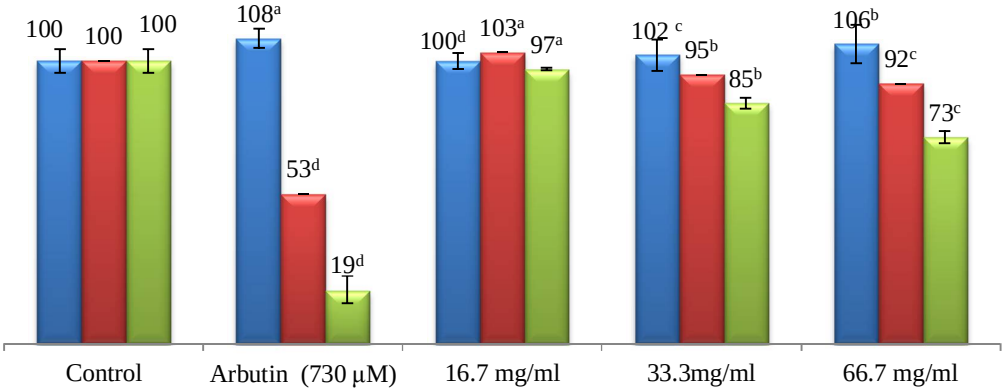

Figure 2 The decreasing number of melanin content by TW 14
: cell viability, : melanin intercellular content,
: melanin extra cellular content

The treatment with goat milk fermentation with lactic acid bacteria starter is not toxic to the cell because the cell viability higher than control (Fig. 2). Through the it could inhibit tyrosinase on diphenolase and decreased inhibitory activity on formation of extracelluler melanin of samples 33.3 mg/ml (15%) . sample withof sample of 66.7 mg/ml resulted decreasing decreasing melanin intracelluler (27%). The effect of lactic acid using *Lactobacillus plantarum* TWK10-fermented extract of soybean milk found that it had higher fungi tyrosinase inhibitory activity and melanin production in B16F0 melanosite compared to non-fermented soybean milk [3].

4. CONCLUSION

Fermented milk with and without lactic acid bacteria (TW 14) act as melanin inhibitor enzimatically of which L-DOPA substrate. Fermented milk with starter had significantly higher effect in melanin inhibition at B16F0 culture cell compared to milk fermented without additional of starter.

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